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Influence of Vitamin D on Fecal Shedding of *Escherichia coli* O157:H7 in Naturally Colonized Cattle†

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ABSTRACT

Three experiments were conducted to evaluate the influence of vitamin D on fecal shedding of *Escherichia coli* O157:H7 in cattle. In the first experiment, two groups of cattle (beef and dairy) were assigned to a control treatment or to receive 0.5×10^6 IU vitamin D per day via oral bolus for 10 days. Fecal samples were collected before and throughout the dosing period for culture of *E. coli* O157:H7. No differences were observed for fecal shedding of *E. coli* O157:H7 among treatments for either beef or dairy animals. Serum concentrations of vitamin D were markedly higher ($P < 0.0001$) in treated beef cattle but only tended to be higher ($P = 0.09$) in the dairy cattle. In the second experiment, three successive vitamin D dosages (2,400, 4,800, and 9,600 IU/day; 14 days each) were administered to 14 dairy steers (7 steers served as controls), fecal samples were collected daily, and serum samples were collected weekly throughout the 42-day experimental period. No significant differences in fecal prevalence or serum vitamin D concentrations were observed for any of the vitamin D dosages. A third experiment sampled feedlot cattle (winter and summer) to determine whether serum vitamin D concentrations were correlated with fecal shedding of *E. coli* O157:H7. A fecal sample and a blood sample were obtained in each season from 60 randomly selected animals (total of 120 fecal samples and 120 corresponding blood samples). As expected, season was highly correlated ($r = 0.66$) with serum vitamin D concentration with higher concentrations ($P < 0.01$) observed in the summer. *E. coli* O157:H7 prevalence (percentage of positive samples) was not highly correlated ($r = 0.16$) with season, although the correlation tended to be significant ($P = 0.08$). The proportion of cattle shedding *E. coli* O157:H7 was 16.7 and 6.7% for the summer and winter collections, respectively. Results of this research do not support a correlation between vitamin D intake and *E. coli* O157:H7 shedding in cattle.

Escherichia coli O157:H7 is found primarily in ruminants *(2),* and fecal shedding of this pathogen typically is more prevalent during the summer months *(3, 13)*. We hypothesized that this seasonal variation is due to the animals' physiological responses to changing day length *(6)*. Findings from previous research conducted in our laboratory with both naturally and experimentally infected cattle and sheep supported this hypothesis and suggested that the hormones melatonin, triiodothyronine, and thyroxin may be involved in the response *(4–6, 12)*.

Cattle derive vitamin D from both dietary sources (D_2 or D_3) and UV light conversion of 7-dehydrocholesterol in the skin (D_3). These molecules are then converted to 1,25-$(OH)_2D$ (responsible for most of the biological activity of vitamin D) and its final metabolite 1,25-$(OH)_2D_3$ *(8, 10)*. One of the three major target tissues of vitamin D is the intestine, where vitamin D stimulates facilitated transport of Ca and P across the intestinal brush border *(14)*. Vitamin D, also known as the ''sunshine'' vitamin, is higher in the serum of cattle during the summer months *(7)*. The seasonal increase in serum vitamin D concentrations and its presence and active role in the intestine led us to hypothesize that vitamin D plays a role in the seasonal prevalence of *E. coli* O157:H7. The objective of the current research was twofold: (i) examine the effect of supplemental vitamin D on fecal shedding of *E. coli* O157:H7 in naturally colonized cattle and (ii) examine serum vitamin D concentrations and fecal *E. coli* O157:H7 prevalence in feedlot steers when serum vitamin D concentrations are at their highest (summer) and lowest (winter).

MATERIALS AND METHODS

Experiments I and II were conducted at the laboratory facilities of the U.S. Department of Agriculture Food and Feed Safety Research Unit Laboratory (College Station, TX). Care, use, and handling of experimental animals were preapproved by the Animal Care and Use Committee of the Food and Feed Safety Research Laboratory. For both experiments, cattle were housed

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in a single large outdoor pen with access to shade. Before initiation of treatments, all cattle were gradually adapted over a 14-day period to an diet with an 80:20 ratio of concentrate:forage fed at 2.5% of body weight (BW) daily each morning after sample collection. Water and salt were provided for ad libitum consumption.

Experiment I. Fourteen crossbred beef steers (mean BW, 225 kg) and 12 Holstein dairy steers (mean BW, 454 kg) were used for this experiment, which was conducted in the fall of the year (September and October). After adaptation to the diet, 13 steers were randomly assigned to each of two treatment groups, with each cattle type (beef or dairy) equally represented in each treatment: control steers were treated orally with a empty gelatin capsule, and steers in the vitamin D_3 group were given 0.5×10^6 IU vitamin D_3 (Nutra Blend LLC, Neosho, MO) orally in a gelatin capsule. In the first phase of the experimental period, fecal samples were collected via rectal retrieval daily for 6 days and cultured qualitatively for *E. coli* O157:H7 as described below. During the second phase, treatments were administered, and fecal samples were collected daily for 10 days. Blood samples were collected via jugular venipuncture for determination of serum vitamin D concentration as described below 1 day after the last treatment administration. Steers were run through a squeeze chute daily (0700 h) for sample collection and treatment administration.

Experiment II. Fourteen Holstein steers (mean BW, 150 kg) were used in this experiment conducted from the end of March through the first of May. The concentrate portion of the diet was specially formulated not to contain any added vitamin D supplement; the only vitamin D was that naturally present in the feed ingredients. Fecal samples were collected four times during the 2-week adaptation period to confirm that all animals were naturally colonized with and shedding *E. coli* O157:H7. Cattle were randomly assigned to control (empty gelatin capsule) or vitamin D treatment groups; oral treatments were administered daily. Three increasing dosages of vitamin D were used in the experiment. The initial dosage of 2,400 IU/day is a supplementation rate commonly used in the cattle feeding industry and was administered for the first 14 days of the experiment. The dosage was increased to 4,800 IU/day for 14 days followed by 9,600 IU/day for the final 14 days of the experiment. During the dosing period, fecal samples were collected daily via rectal palpation and cultured (quantitatively and qualitatively) for *E. coli* O157:H7 as described below. Serum samples were collected weekly via jugular venipuncture for determination of vitamin D concentrations, also as described below.

Feedlot study. Samples were collected twice, once in February (winter collection) and once in August (summer collection) of 2010, at a commercial feedlot in the southwestern United States. A fecal sample (retrieved rectally into a sterile palpation sleeve) and a blood sample (obtained via jugular venipuncture) were obtained from 60 randomly selected steers at each sampling time (120 fecal samples and 120 corresponding blood samples). All cattle were restrained in a squeeze chute for a routinely scheduled working procedure, and our samples were collected at this time; thus, the cattle were not processed specifically for our collections. Fecal samples were shipped overnight to our laboratory (College Station, TX) for bacterial culture as described below. Blood samples were allowed to clot at room temperature and then centrifuged ($1,500 \times g$ for 15 min), and serum was decanted, frozen, and shipped to our laboratory for determination of serum vitamin D concentrations.

Bacterial culture and isolation. All fecal samples were processed the day of collection (experiments I and II) or the day following collection (feedlot experiment) and cultured for *E. coli* O157:H7 as described previously *(11)* and modified *(1)*. Only qualitative analyses were conducted on samples in experiment I, whereas both qualitative and quantitative analyses were conducted on samples in experiment II and the feedlot experiment. Putative *E. coli* O157 colonies (flat mauve colonies lacking a distinct center) were confirmed as the O157:H7 serotype using the Reveal microbial screening test (Neogen Corp., Lansing, MI) according to the manufacturer's instructions. Enumeration of *E. coli* O157:H7 was performed by direct plating of a mixture of feces and tryptic soy broth with phosphate (before enrichment) onto ntChrom-O157 agar using a commercially available spiral plater (Spiral Biotech Autoplate 4000, Advanced Instruments, Inc., Norwood, MA). Plates were incubated overnight at 42°C. After confirmation of one or two colonies as *E. coli* O157:H7, all remaining colonies were counted and the population was calculated.

Serum vitamin D determination. Serum concentrations of vitamin D (25-hydroxy vitamin D) were determined using a commercially available enzyme-linked immunosorbent assay kit (Immuno Diagnostics Systems Inc., Fountain Hills, AZ). Serum samples were diluted five times in sterile water and then analyzed following the manufacturer's instructions. Vitamin D concentration was determined by measuring the optical density at 450 to 650 nm ($OD_{450–650}$) using a SPECTRA MAX microplate reader (Molecular Devices, Sunnyvale, CA).

Statistical analyses. For experiments I and II, daily fecal shedding data of *E. coli* O157:H7 was noted from all animals throughout the entire experimental period. However, because of the sporadic nature of shedding and the number of animals utilized in this research, the data were insufficient to analyze by day and therefore were pooled across days by cattle type (beef or dairy) and are presented by each phase of the experiment (6-day pretreatment period and 10-day treatment period) in experiment I. For experiment II, daily fecal shedding data were pooled for each 14-day period and are presented by vitamin D dose and across all doses. Few animals shed quantifiable levels of *E. coli* O157:H7, and those levels were low ($\leq 10^3$ CFU) and similar among treatments; therefore, these data were analyzed as incidence of positive samples via direct plating, as were all fecal shedding data, using the PROC GLIMMIX procedure of SAS version 8.02 (SAS Institute Inc., Cary, NC). Results are presented as the percentage of fecal samples positive for *E. coli* O157:H7. Serum vitamin D concentrations were analyzed using the Proc Mixed procedure. Differences among means were considered significant at the 5% level.

For the feedlot experiment, Spearman rank correlation coefficients were generated using the PROC FREQ procedure of SAS to examine correlations between *E. coli* O157:H7 shedding and serum vitamin D concentrations as influenced by season. The PROC MIXED procedure was utilized to examine the effect of season, *E. coli* shedding status (positive or negative), and *E. coli* shedding status within season on vitamin D concentrations.

RESULTS

Experiment I. Fecal shedding and serum vitamin D concentration data are presented by cattle type in Figures 1 (beef steers) and 2 (Holstein steers). No differences ($P > 0.10$) in the percentage of beef or dairy steers shedding *E. coli* O157:H7 were observed during phase 1 of the experiment.

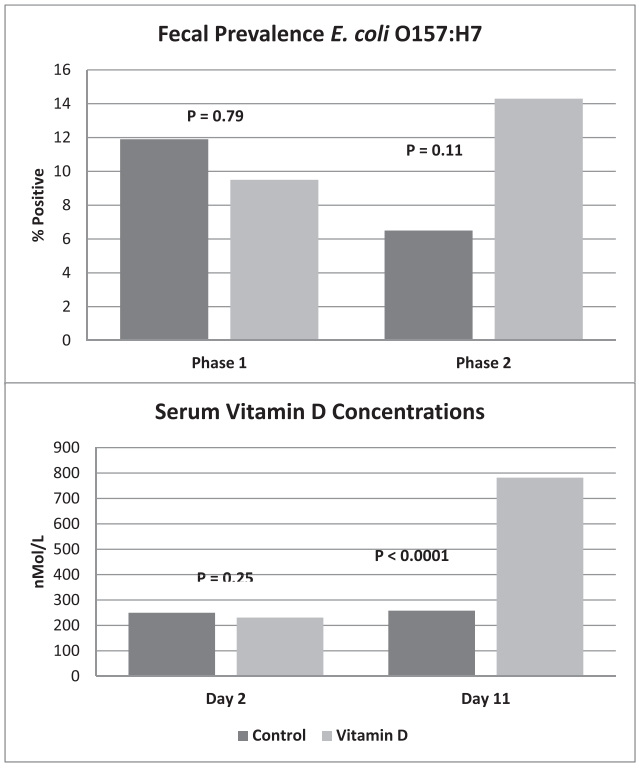

FIGURE 1. *Fecal prevalence of* E. coli *O157:H7 (percentage of positive fecal samples) during a 6-day pretreatment (phase 1) and 10-day treatment (phase 2), and serum concentrations of vitamin D after 2 and 11 days of vitamin D administration (0.5 × 10^6 IU/day) in beef steers (experiment I).*

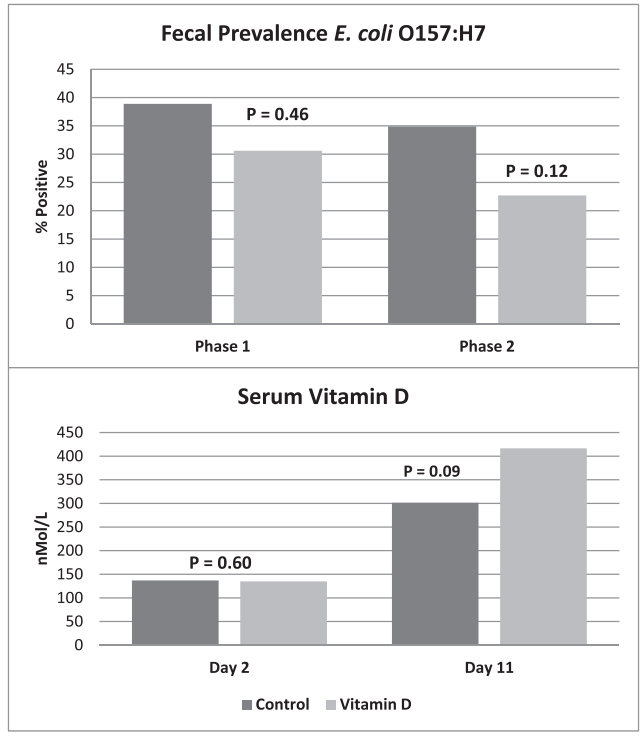

FIGURE 2. *Fecal prevalence of* E. coli *O157:H7 (percentage of positive fecal samples) during a 6-day pretreatment (phase 1) and 10-day treatment (phase 2) and serum concentrations of vitamin D after 2 and 11 days of vitamin D administration (0.5 × 10^6 IU/day) in Holstein dairy steers (experiment I).*

During the 10-day period of vitamin D administration (phase 1), fecal shedding of *E. coli* O157:H7 declined in the control beef steers (reduced by 5.4%), whereas prevalence increased in treated beef steers (4.8%) compared with phase 1 averages; however, these differences between treatment groups were not significant. Fecal shedding in the dairy steers decreased in both treatment groups when comparing phase 1 to phase 2 (4.0 and 7.9% reductions for control and vitamin D treatment groups, respectively).

Serum vitamin D concentrations were not different ($P > 0.20$) in beef steers after 2 days of vitamin D treatment but were markedly increased ($P < 0.0001$) after 10 days of treatment. Serum concentrations of vitamin D were not different ($P > 0.10$) before vitamin D treatment but tended to be higher ($P = 0.10$) in treated than in control dairy steers after treatment.

Experiment II. The number and percentage of fecal samples culture positive (by direct plating and after enrichment) for *E. coli* O157:H7 is presented collectively for each phase (vitamin D dosage) of the experiment (Table 1). Few samples were *E. coli* O157:H7 positive by direct plating, all of which had low levels of the pathogen (2 to 3 log CFU/g of feces). Numerically, fewer samples were culture positive via direct plating in animals administered 2,400 and 9,600 IU of vitamin D and when the data were combined across treatments. However, statistical analysis revealed no significant differences by direct plating for any of the vitamin D dose groups or when the data were combined across dosages. Similarly, no differences ($P > 0.20$) were found in the number of samples positive for *E. coli* O157:H7 after enrichment and immunomagnetic separation for any of the three vitamin D dosages or when the data were combined across dosages. The percentage of positive samples decreased during the experimental period, averaging 54 and 50% for control and vitamin D treatments, respectively.

Serum vitamin D concentrations are presented by week of the experiment and by treatment (Table 2). No treatment differences ($P > 0.20$) were observed for any of the vitamin D dosages or when the data were combined across days and for the control versus vitamin D treatment comparisons.

Feedlot experiment. As expected, season was highly correlated ($r = 0.66$, $I < 0.01$) with serum vitamin D concentration; higher concentrations were found observed in the summer samples. The *E. coli* O157:H7 status of an animal (i.e., positive or negative) was not correlated with serum vitamin D concentration. Spearman rank correlation coefficients did not differ from zero ($P > 0.05$ for both correlations) for samples collected in either summer ($r = 0.04$; 95% confidence limit [CL] = −0.2, 0.3) or winter ($r = 0.16$; 95% CL = −0.02, 0.3). The proportion of cattle shedding *E. coli* O157:H7 was 16.7 and 6.7% for the summer and winter samples, respectively. These qualitative *E. coli* O157:H7 results were obtained after enrichment and immunomagnetic separation. None of the 60 summer samples and only one winter sample had quantifiable *E. coli* O157:H7, indicating

TABLE 1. *Prevalence of* E. coli *O157:H7 in fecal samples collected from Holstein dairy steers administered 2,400, 4,800, or 9,600 IU vitamin D per day for 14 days (experiment II)*[a]

Day	Vitamin D dose (IU) per day	Direct plating					Enrichment and IMS				
		Control		Vitamin D			Control		Vitamin D		
		n	%	*n*	%	*P* > *F*	*n*	%	*n*	%	*P* > *F*
1–14	2,400	6/98	6.1	2/98	2.0	0.15	66/98	67.4	61/98	62.2	0.45
15–28	4,800	2/98	2.0	3/98	3.1	0.65	58/98	59.2	50/98	51.0	0.25
29–42	9,600	3/98	3.1	1/98	1.0	0.31	35/98	35.7	36/98	36.7	0.88
1–42	All doses	11/294	3.7	6/294	2.0	0.22	159/294	54.1	147/294	50.0	0.32

[a] Values are the number of positive samples/number of samples tested. The percentage of positive samples also is presented because of low *E. coli* O157:H7 levels. Fecal samples were plated directly or were enriched and subjected to immunomagnetic separation (IMS) and then plated for qualitative analysis.

that although this pathogen was present it was in fairly low levels in the feces. Figure 3 shows the influence of season, *E. coli* O157:H7 shedding status (positive or negative), and *E. coli* O157:H7 shedding status within season on serum vitamin D concentrations. Only season significantly influenced vitamin D concentrations. Vitamin D was not different among animals shedding or not shedding *E. coli* O157:H7 when examined within each season or when shedding data were combined across seasons.

DISCUSSION

Although not significant, results of experiment I suggest (weakly) that vitamin D treatment may have influenced fecal shedding of *E. coli* O157:H7 in cattle. Among the beef cattle, more animals shed *E. coli* O157:H7 after vitamin D administration with a corresponding highly significant increase in serum concentrations of vitamin D. In contrast, administration of vitamin D to the Holstein dairy steers did not affect fecal shedding, with only a modest nonsignificant increase in serum concentrations of vitamin D observed in treated animals when compared with control animals; increased concentrations were found in both treatments on day 11. Reasons for this increase across treatments is unknown. This difference in fecal shedding in phase 2 is similar to the difference among treatment groups observed in phase 1 before vitamin D administration and probably is not a direct effect of vitamin D treatment. Differences in response between the two groups of cattle are probably a function of body weight; the dairy cattle averaged 454 kg compared with 225 kg for the beef cattle, and both groups of cattle received the same dose of vitamin D. Serum concentrations in the treated dairy cattle were approximately half those in the treated beef cattle, whereas concentrations in control beef and dairy cattle were similar.

Based on results of experiment I, a second experiment was designed to further evaluate the influence of vitamin D on fecal shedding and populations of *E. coli* O157:H7 using concentrations of vitamin D more closely approximating supplementation rates used in the cattle industry. The lack of effect of vitamin D administration on *E. coli* O157:H7 and serum vitamin D concentrations in the second experiment is most likely a reflection of the vitamin D doses utilized in the two studies. In the first experiment, we administered 0.5×10^6 IU/day for 8 days. This dose was based on previous research on the effect vitamin D supplementation on postmortem tenderization of beef *(9)*. The authors reported that this level of supplementation for 9 days improved tenderness without adversely affecting performance or tissue residues. In comparison, the initial vitamin D dose of 2,400 IU/day utilized in experiment

TABLE 2. *Serum vitamin D concentrations in Holstein steers after treatment with 2,400, 4,800, or 9,600 IU vitamin D per day for 42 days (experiment II)*[a]

Day	Vitamin D dose (IU) per day	Vitamin D concn (nmol/liter)			*P* > *F*
		Control	Vitamin D	SEM	
7	2,400	185	176	8.6	0.47
14	2,400	196	183	11.2	0.43
28	4,800	184	172	14	0.54
35	9,600	179	179	10.9	0.98
42	9,600	202	189	8.3	0.31
All days	All doses	189	180	7.6	0.46

[a] Each dosage was administered for 14 days.

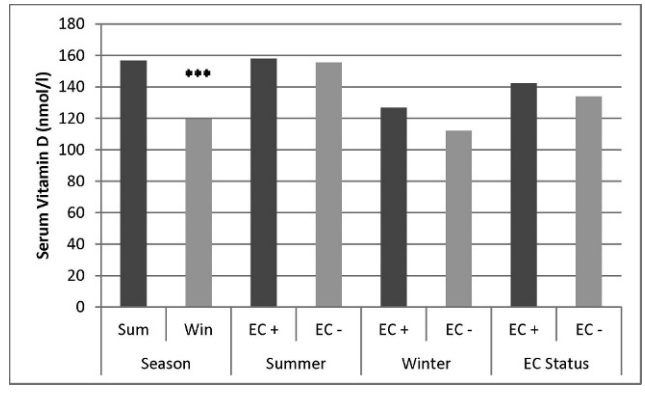

FIGURE 3. *Serum vitamin D concentrations (nanomoles per liter) in feedlot steers as affected by season of sample collection (Sum, summer; Win, winter).* E. coli *O157:H7 shedding status (EC+, fecal sample positive; EC−, fecal sample negative) was determined within and across seasons. Significant differences are denoted by* *** $P < 0.01$.

II was based on the current feeding rate of vitamin D employed in the cattle industry. We hypothesized that if this lower dose was able to produce similar responses in fecal shedding, as observed in the first experiment, then complete removal of vitamin D from the ration for feedlot cattle in the summer would have a beneficial effect on *E. coli* O157:H7 populations or prevalence. Serum vitamin D concentrations have been reported as higher in the summer because of increased day length and light intensity *(7)*; therefore, we speculated that the removal of supplementary vitamin D would be offset by the seasonal increase and there would not be any adverse effects on performance or carcass quality. The 2,400, 4,800, and 9,600 IU doses failed to elicit any change in *E. coli* O157:H7 prevalence; thus, it appears unlikely that our scenario of eliminating vitamin D supplementation during the summer months would be of much use for reducing pathogen prevalence.

The 14-day supplementation period used in experiment I may not have been long enough to evoke the physiological changes that occur in animals exposed to gradual changes in day length and subsequent vitamin D concentrations. However, the combined vitamin D doses were administered for 42 consecutive days, which makes the length of the experiment an unlikely explanation for the results. Previous research revealed an influence of lighting *(6),* exogenous melatonin *(5),* and thyroid hormones *(4)* on *E. coli* O157:H7 in cattle and subsequently led us to believe that the physiological changes that occur in these animals in response to day length and influence *E. coli* O157:H7 populations are likely multifaceted and involve a cascade of events. Possibly the high dose of vitamin D administered in experiment I was effective in initiating this hypothesized chain of events, whereas the dose and/or duration of treatment used in the second experiment was insufficient to evoke a similar response in *E. coli* O157:H7 prevalence. This possibility is supported by the lack of a treatment effect on serum concentrations of vitamin D observed in experiment II as compared with experiment I. If the summer elevation in vitamin D concentration is involved in the concomitant increased prevalence of *E. coli* O157:H7, then any change in *E. coli* prevalence should be associated with a change in serum vitamin D concentrations. This association was observed in the first but not the second experiment and may be an indication that our methodology for vitamin D treatment, but not our hypothesis, was inadequate in the second experiment and led to the feedlot experiment.

In the feedlot experiment, we hoped to determine whether serum vitamin D concentrations were correlated with fecal shedding of *E. coli* O157:H7. Season was highly correlated with serum vitamin D concentration in these feedlot cattle, as has been reported previously *(7),* but whether the animal was shedding *E. coli* O157:H7 was not correlated with season. A larger total number of samples may have subsequently increased the number of *E. coli* O157:H7–positive samples, increasing the sensitivity of the statistical analysis and revealing stronger correlations. Only four fecal samples were positive for *E. coli* O157:H7 in the winter, and thus may have limited the analysis. However, the collection of blood samples requires handling and restraint of the cattle and was accomplished during other handling procedures and not specifically for this experiment. In an effort to minimize our intrusion into the normal cattle working routines on this feedlot, we chose to sample 60 animals per collection time. This number has been sufficient to detect significant differences in cattle in commercial production operations in previous studies conducted by our laboratory.

The results of these three experiments suggest a possible, albeit slight, role of vitamin D in the fecal shedding of *E. coli* O157:H7 in cattle. Results of our previous research concerning the seasonality of *E. coli* O157:H7 shedding in cattle support the hypothesis that physiological responses within the animal to changing day length may contribute to the fluctuations in shedding of this pathogen and likely involve a cascade of events and multiple hormones (including vitamin D) and/or other compounds yet to be determined.

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